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A polymorphism in myostatin influences puberty but not fertility in beef heifers, whereas μ-calpain affects first calf birth weight[1]

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ABSTRACT: The use of genetic markers to aid in selection decisions to improve carcass and growth characteristics is of great interest to the beef industry. However, it is important to examine potential antagonistic interactions with fertility in cows before widespread application of marker-assisted selection. The objective of the current experiment was to examine the influence of 2 commercially available markers currently in use for improving carcass traits, the myostatin (*MSTN*) F94L and μ-calpain (*CAPN1*) 316 and 4751 polymorphisms, on heifer development and reproductive performance. In Exp. 1, beef heifers (n = 146) were evaluated for growth and reproductive traits over a 3-yr period to determine if these polymorphisms influenced reproductive performance. In Exp. 2, heifers representing the 2 homozygous genotypes for the *MSTN* F94L polymorphism were slaughtered on d 4 of the estrous cycle and reproductive tracts were collected for morphological examination. In Exp. 1, there was a tendency (P = 0.06) for birth BW to be affected by *MSTN* with the Leu allele increasing birth BW in an additive fashion. Additionally, *MSTN* significantly affected the proportion of pubertal heifers by the start of the breeding season ($P < 0.05$) with the Leu allele additively decreasing the proportion pubertal; however, this did not result in a delay in conception or a decrease in pregnancy rates during the first breeding season ($P > 0.15$). The GT haplotype of *CAPN1*, which was previously associated with decreased meat tenderness, was associated with an additive decrease in birth BW of the first calf born to these heifers ($P < 0.05$). In Exp. 2, there were no differences between the *MSTN* genotypes for gross or histological morphology of the anterior pituitary, uterus, or ovaries ($P > 0.05$). From these results, we concluded that the *MSTN* F94L and *CAPN1* polymorphisms can be used to improve carcass traits without compromising fertility in beef heifers. The influence of these markers on cow performance and herd life remains to be determined. While the delay in puberty associated with the *MSTN* F94L polymorphism did not negatively impact reproductive performance in heifers, caution should be used when combining this marker with other markers for growth or carcass traits until the potential interactions are more clearly understood.

Key words: fertility, heifer development, μ-calpain, myostatin, puberty

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[1]Names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of names by the USDA implies no approval of the product to the exclusion of others that may also be suitable.

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INTRODUCTION

The use of genetic markers to improve growth and carcass traits may have antagonistic impacts on the reproductive performance of the cow herd. Collis et al. (2012) examined this relationship in 7 genes associated with production traits, and reported that, in many cases, the allele associated with favorable production traits was not favorable for reproductive traits. For example, the allele of *CAPN1* that was associated with increased tenderness was also associated with longer postpartum anestrous intervals in cows. In another study, there were antagonistic genetic correlations of residual feed intake and carcass color in steers with age at puberty in heifers (Johnston et al., 2009).

Growth and differentiation factor-8 (*GDF-8*), also known as myostatin (*MSTN*), is a member of the transforming growth factor-β (*TGF*-β) superfamily of growth factors. Several polymorphisms identified in the *MSTN* gene in beef cattle are associated with increased muscling, making this gene an attractive candidate for marker-assisted selection (Grobet et al., 1997). Phocas (2009) reported that while the *MSTN* Q204X polymorphism increased the muscling score of heifers, it had no influence on fertility in Charolais cattle. The *MSTN* F94L polymorphism was associated with LM area as well as quantity and composition of fat in Limousin × Wagyu steers (Alexander et al., 2009), but there are no reports on the influence of this polymorphism on reproductive traits. Before the beef industry adopts the *CAPN1* and *MSTN* F94L markers into selection decisions, it is important to understand how these polymorphisms are associated with reproductive performance in the cow herd. Therefore, the objective of this study was to determine the influence of these polymorphisms on heifer development and reproductive performance to test the hypothesis that selection for the allele that was favorable for production traits would negatively impact reproductive performance in beef heifers.

MATERIALS AND METHODS

Genotyping

All procedures were approved by the U.S. Meat Animal Research Center (**USMARC**) Animal Care and Use Committee (number 47-F-002). Blood samples were collected by jugular venipuncture from heifers into 10-mL syringes with 4% EDTA. Blood was frozen at –20°C until DNA was extracted. Samples of DNA were extracted from blood for heifers and young bulls or semen for historical sires used in the selection phase of the study. Extraction of DNA was performed using a Qiagen QIAmp DNA mini blood kit (Qiagen, Valencia, CA). Genotyping was performed using a primer extension method with mass spectrometry-based analysis of the extension products on a MassArray system as suggested by the manufacturer (Sequenom, Inc., San Diego, CA) and described previously (Stone et al., 2002; Alexander et al., 2009; Cushman et al., 2013). When necessary, genotype assays were repeated to resolve missing genotypes.

Experiment 1

Cattle Population. The Meat Animal Research Center (**MARC**) I (one-eighth Angus, one-eighth Hereford, one-fourth Braunvieh, one-fourth Limousin, and one-fourth Charolais) composite population used in this study originated from the USMARC calving ease population reported by Bennett (2008) and was subsequently selected to increase the frequency of the Leu allele of the *MSTN* F94L SNP identified by Grobet et al. (1998), which substitutes leucine (Leu) for phenylalanine (Phe) at the 94th amino acid in *MSTN* (BTA2; rs110065568). Additionally, this population was selected to increase and equalize 2 haplotypes within the *CAPN1* gene. Markers *CAPN1*_316 (BTA29; rs17872000; Page et al., 2002), segregating C and G alleles, and *CAPN1*_4751 (BTA29; rs17872050; White et al., 2005), segregating C and T alleles, were used to define haplotypes for selection. The *CAPN1* CC and GT haplotypes were chosen for selection in this population because, among haplotypes with >1% frequency, they were the most divergent for 14-d Warner-Bratzler shear force (White et al., 2005).

Phenotypes. After 3 yr of selection to attain the frequency of genotypes desired (Table 1), 3 birth yr (spring 2007 to 2009) of MARC I replacement heifers (n = 146) were evaluated for growth and reproductive performance. Dams were 2 to 12 yr of age but age was defined as 2, 3, 4, and ≥5 for analysis. Heifers were weighed at birth and weaning (average age = 161 d; SD = 18.7 d). After weaning, heifers were moved to the USMARC feedlot and allocated by *MSTN* and *CAPN1* genotypes to 1 of 2 development protocols. The first protocol was the standard replacement heifer diet, where heifers were fed to have a uniform gain throughout development from weaning to breeding with a target weight of 65% of mature weight at the beginning of the breeding season. The second development protocol was a 2-step development described previously (Freetly et al., 2015). Briefly, gain in BW was reduced while the heifers were expected to be initiating reproductive cycles, and then BW gain was increased for the second half of the development program to achieve the same target weight as the first protocol. The genotypes for *MSTN* and *CAPN1* were equally stratified across dietary groups.

Table 1. Heifer genotype frequencies for *MSTN* F94L genotypes by haplotypes for *CAPN1_316* with *CAPN1_*4751 in a population selected to equalize their frequencies

CAPN1 diplotypes[1]	*MSTN* genotypes			
	Phe:Phe	Phe:Leu	Leu:Leu	Total
CC:CC	9	28	11	48
CC:GT	29	29	15	73
GT:GT	10	11	4	25
Total	48	68	30	146

[1]Haplotypes of *CAPN1_316* allele and *CAPN1_4751* allele are CC and GT.

From 11 to 13 mo of age, heifers were evaluated monthly by ultrasonography to determine pubertal status (Cushman et al., 2009; Fortes et al., 2010; Eborn et al., 2013). Briefly, all heifers were weighed and submitted for ultrasonographic evaluation of the ovaries for the presence of a corpus luteum (**CL**) at 11 mo of age (average age = 326 d). At 12 mo of age (average age = 368 d), all heifers were weighed and those that were not identified with a CL at 11 mo of age were once more submitted for ultrasonographic evaluation to determine the presence or absence of a CL. At 13 mo of age (average age = 393 d), all heifers that had not previously been identified with a CL were submitted for ultrasonographic evaluation to determine the presence or absence of a CL. Heifers were weighed again at 14 mo of age before going to breeding. In the first 2 yr of the study, heifers were observed twice daily for behavioral estrus during the first 21 d of the breeding season and artificially inseminated 12 h after observed estrus. After the 21 d estrus detection period, all heifers were placed with bulls for an additional 39 d. In the third year of the study, heifers were placed with bulls for 60 d of natural service. Approximately 70 d after the end of the breeding season, heifers were examined by ultrasonography to determine pregnancy status. For pregnant heifers, first calf performance was analyzed. Traits for the first calf were Julian calving date, whether a calf was weaned and its age, and calf BW at birth and weaning. Calf BW at weaning was analyzed 2 ways by either adjusting for age at weaning or ignoring calf age.

Experiment 2

Cattle Population. Pubertal heifers that were homozygous for the Phe allele (n = 5) or homozygous for the Leu allele (n = 5) of the F94L *MSTN* genetic marker were administered 2 injections of Lutalyse (5 cm^3, intramuscular injection; Pfizer Animal Health, Madison, NJ) given 11 d apart to synchronize the estrous cycles. At the second injection, heifers were fitted with Estrotect patches (Western Point Inc., Apple Valley, MN) and observed twice daily to determine onset of behavioral estrus. Heifers were slaughtered on d 4 after estrus. Reproductive tracts were removed, trimmed of excess tissue, and weighed. The anterior pituitary was excised, trimmed, and weighed. A sample of subcutaneous adipose was removed from the tail head and frozen in liquid nitrogen for subsequent analysis of chemerin (*RARRES2*) mRNA expression using a previously validated real-time reverse-transcription PCR (**RT-PCR**) assay because mRNA amounts in adipose were a potential biomarker of BCS and adipose function (Lindholm-Perry et al., 2012). Samples were run in duplicate, and the average intra-assay CV was 7.3 and 10.1% for chemerin and tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, γ polypeptide (*YWHAG*), respectively.

In the laboratory, the ovaries were removed from the reproductive tract, measured, and weighed. The diameter of the uterine horns and the endometrium were measured for both the left and right horn 1 cm anterior to the bifurcation as previously described (Cushman et al., 2013). The CL were removed from the ovaries and weighed. All surface follicles were classified as small (<5 mm) or medium (5–10 mm) and counted. Follicular fluid was aspirated from all small follicles and pooled. The pooled follicular fluid was centrifuged at 1,000 × g for 5 min at room temperature, removed from the granulosa cell pellet, and frozen. Medium follicles were measured and aspirated and individual follicular fluid was centrifuged at 1,000 × g for 5 min at room temperature, removed from the granulosa cell pellet, and frozen for estradiol determination by RIA. The individual granulosa cell pellets of medium follicles were weighed. The theca was dissected from individual medium follicles and weighed. A cross-section of 1 ovary approximately 5 mm thick was fixed in 10% neutral buffered formalin and embedded in paraffin for histological evaluation.

Morphometric Evaluation of the Ovaries. For each heifer, 5 representative 6-μm sections that were separated by a minimum of 10 sections to avoid counting the same follicles twice were placed on slides and stained with hematoxylin and eosin. A trained technician that was uninformed as to genotype evaluated the sections to determine the number of primordial, primary, and secondary follicles (van Wezel and Rodgers, 1996; Fortune et al., 2000; Cushman et al., 2001). Briefly, primordial follicles were defined as an oocyte surrounded by a single layer of squamous pregranulosa cells. Primary follicles were defined as an oocyte surrounded by a single layer of cuboidal granulosa cells, and secondary follicles were defined as an oocyte surrounded by 2 or more layers of granulosa cells.

Follicular Fluid Estradiol RIA. Follicular fluid concentrations of estradiol-17β were analyzed using

methodology similar to that described by Perry and Perry (2008). Follicular fluid was not extracted but was serially diluted in assay buffer 1:100, 1:1,000, 1:10,000, and 1:100,000 before being added to the reaction tubes. Duplicates of each dilution (100 µL) were incubated with 100 µL of estradiol-17β antisera (MP Biomedical, Santa Anna, CA; 1:450,000 vol/vol dilution) at 37°C for 5 min followed by 1 h at 4°C. Following incubation, 100 µL of [^{125}I] estradiol-17β (MP Biomedical; 2,000 µCi/µg; adjusted to 5,000–6,000 counts per minute) was added to each tube. Tubes were incubated at 4°C for 20 h. Bound and free estradiol was separated by addition of 0.5 mL dextran-coated charcoal solution (10 min incubation) followed by centrifugation at 3,000 × *g* for 10 min at room temperature. Supernatants were counted in a γ counter for 5 min per tube. Intra- and interassay CV for estradiol-17β assays were 3.1 and 6.0%, respectively, and assay sensitivity was 0.5 pg/mL.

Table 2. Heifer performance and first calf performance traits summary

Trait	Mean	SD	Minimum	Maximum
Own performance traits				
Birth BW, kg	35.8	5.0	23.6	54.4
Weaning BW, kg	190.8	23.3	130.6	252.2
11 mo BW, kg	304.9	37.2	185.1	403.7
11 mo pubertal	0.521	0.501	0.000	1.000
12 mo BW, kg	340.1	38.7	206.8	455.4
12 mo pubertal	0.842	0.366	0.000	1.000
13 mo BW, kg	370.7	42.1	243.1	513.5
13 mo pubertal	0.884	0.322	0.000	1.000
First season pregnancy	0.904	0.295	0.000	1.000
First calf performance traits				
First calf weaning rate	0.834	0.373	0.000	1.000
Julian calving date, d	91.4	13.9	64.0	136.0
Birth BW, kg	34.6	4.9	20.9	47.2
Weaning BW, kg	198.4	32.2	90.7	289.4

Statistical Analyses

Experiment 1. All genetic marker and pedigree information was processed through GenoProb, a software program that implements algorithms described by Thallman et al. (2001a,b). GenoProb identifies genotyping errors and orders genotypes based on genotypes of relatives. GenoProb identified genotypes that were used for *MSTN* analysis. GenoProb most likely orders genotypes according to parent of origin and the GenoProb ordered genotypes were used to create the haplotypes for *CAPN1* analysis (Table 1). Candidate evaluation phase heifers were removed from analysis when GenoProb ordered genotypes indicated an undesired haplotype present for *CAPN1* ($n = 7$).

Heifer performance for continuous traits were analyzed with PROC MIXED of SAS (SAS Inst. Inc., Cary, NC) and binary traits were analyzed with PROC GLIMMIX of SAS with a Logit link function. Fixed effects modeled were year of birth (2007, 2008, or 2009), age of dam (2, 3, 4, or 5+), postweaning development protocol (constant or 2-stage), *MSTN* genotype (Phe:Phe, Phe:Leu, or Leu:Leu), *CAPN1* haplotype genotype (CC:CC, CC:GT, or GT:GT), and covariate of age of heifer (d). The residual was modeled to be approximately $N(0,\sigma_e^2)$. The additive effects of the GT haplotype for *CAPN1* and Leu allele for *MSTN* were determined using the ESTIMATE statement with coefficients of –0.5, 0, and 0.5 (e.g., –0.5 × Phe:Phe + 0 × Phe:Leu + 0.5 × Leu:Leu for *MSTN*). Similarly, dominance effects of GT and Leu were determined using coefficients of –0.5, 1, and –0.5.

One heifer calving twins was removed from the first calf performance trait analyses. This edit and fertility performance levels yielded 128 calving and 121 weaning records. First calf performance traits were analyzed with PROC MIXED of SAS (SAS Inst. Inc.). Fixed effects modeled were calf year of birth (2009, 2010, or 2011), calf sex (bull or heifer), dam postweaning development protocol (constant or 2-stage), dam *MSTN* genotype (Phe:Phe, Phe:Leu, or Leu:Leu), and dam *CAPN1* haplotype genotype (CC:CC, CC:GT, or GT:GT). Because *MSTN* genotype was suggestive for birth BW in dams ($P = 0.06$), first calf birth BW was also analyzed with calf *MSTN* genotype (Phe:Phe, Phe:Leu, or Leu:Leu) added. First calf weaning weight was also analyzed with an additional model that included calf age in days as a covariate.

Experiment 2. Morphological and histological traits were analyzed using the MIXED procedure of SAS with the *MSTN* genotype as a fixed effect. Relative amounts of chemerin mRNA were determined using the 2–ΔΔCT method (Livak and Schmittgen, 2001) with *YWHAG* as our previously validated internal reference gene (Lindholm-Perry et al., 2012) and were analyzed using the MIXED procedure of SAS with genotype as a fixed effect. The relationship between anterior pituitary weight and antral follicle count was examined using the REG procedure of SAS.

RESULTS

Experiment 1. Means and ranges of performance traits for heifers and their first calves are reported in Table 2. Fixed effects other than *MSTN* and *CAPN1* genotypes were included in the statistical models to account for their effects but were not the focus of this study, so their significance and effects will not be discussed here. There was a tendency for *MSTN* genotype

Table 3. Significance (*P*-value) of fixed effects on heifers own performance traits

Trait	Birth year	Dam age	Age[1]	Development	*MSTN*	*CAPN1*
Birth BW	<0.001	0.05	0.14	0.59	0.06	0.42
Weaning BW	<0.01	<0.001	<0.001	0.19	0.36	0.13
11 mo BW	<0.001	0.01	<0.001	<0.001	0.33	0.23
11 mo pubertal	0.54	0.35	<0.001	0.02	<0.001	0.18
12 mo BW	0.13	0.08	<0.001	<0.001	0.41	0.22
12 mo pubertal	0.06	0.21	<0.001	<0.01	0.03	0.36
13 mo BW	0.04	0.07	<0.001	<0.001	0.63	0.60
13 mo pubertal	0.06	0.10	<0.01	0.01	0.03	0.48
First season pregnancy	0.98	0.40	0.93	0.61	0.56	0.63

[1]Age of the animal (d) at time of phenotype collection, except birth BW used Julian birth date; first season pregnancy used age at the beginning of the breeding season.

to affect birth BW in heifers ($P = 0.06$; Table 3). The mode of inheritance was additive ($P = 0.03$), with the Leu allele increasing birth BW 1.31 kg (Table 4). By weaning, there was no difference in BW between the 3 *MSTN* genotypes ($P = 0.36$), and *MSTN* genotype did not influence postweaning BW at any of the 3 ultrasonographic evaluations ($P \geq 0.33$).

The proportion of heifers that were identified with a CL at ultrasonography at 11 mo of age was significantly affected by *MSTN* genotype ($P < 0.001$) with the Leu allele decreasing puberty rate, with very strong evidence of an additive mode of inheritance ($P < 0.001$) but lacking support for dominance ($P = 0.27$; Table 4; Fig. 1A). The significant influence of *MSTN* genotype on proportion of heifers with a CL continued at 12 mo of age ($P = 0.03$), again with support for an additive mode of inheritance ($P < 0.01$; Table 4; Fig. 1B). The proportion that were pubertal still differed by *MSTN* genotype at 13 mo of age ($P = 0.03$), with an additive mode of inheritance ($P = 0.02$). Due to the nonlinear transformation of puberty data, additive effects of *MSTN* on proportion pubertal at 13 mo were –0.026 going from Phe:Phe to Phe:Leu genotype and –0.071 going from Phe:Leu to Leu:Leu genotype. While the 13-mo time point had the strongest support for dominance mode of inheritance ($P = 0.16$; Fig. 1C), dominance estimates at all time points showed a numerical advantage of the *MTSN* heterozygous heifers on puberty. However, *MSTN* genotype of the heifer did not influence ($P = 0.56$) the proportion of heifers that were pregnant at ultrasonographic diagnosis 70 d after the end of the breeding season. Likewise, *MSTN* genotype of the heifer did not influence the proportion of heifers that weaned a calf the following year ($P = 0.77$; Table 5), the Julian day of calving for the first calf ($P = 0.15$), the first calf birth BW ($P = 0.54$), calf weaning BW ($P = 0.35$), or calf weaning BW with calf age effect in the model ($P = 0.71$).

The *CAPN1* haplotypes did not affect heifer performance ($P \geq 0.13$), puberty ($P \geq 0.18$), or fertility ($P = 0.63$) traits (Tables 3 and 5). Other than first calf birth BW ($P = 0.04$; Table 5), the heifer *CAPN1* haplotype also had no influence on first calf performance traits ($P \geq 0.32$). The dam *CAPN1* haplotype had an additive mode of inheritance ($P = 0.01$; Table 6) on first calf birth BW with the GT haplotype decreasing first calf birth BW by 1.5 kg.

Experiment 2. The anterior pituitary weight did not differ between heifers that were homozygous for the Phe allele and heifers that were homozygous for the Leu allele (1.1 ± 0.1 vs. 1.0 ± 0.1 g, respectively; $P = 0.58$). Regression analysis identified a tendency for a positive linear relationship between antral follicle count and anterior pituitary weight (Fig. 2; $P = 0.06$, $r^2 = 0.37$). There was no difference in the weight or size of

Table 4. Least squares means for *MSTN* genotypes on the own performance traits of heifers and significance (*P*-value) of additive and dominance modes of inheritance

	MSTN genotypes			Additive effect of Leu		Dominance effect of Leu	
Trait[1]	Phe:Phe	Phe:Leu	Leu:Leu	*P*-value	Estimate ± SE	*P*-value	Estimate ± SE
Birth BW, kg	34.6	35.0	37.2	0.03	1.31 ± 0.58	0.25	–0.93 ± 0.80
11 mo pubertal, proportion[2]	1.01 (0.732)	0.10 (0.524)	–1.74 (0.149)	<0.001	–1.37 ± 0.34	0.27	0.46 ± 0.42
12 mo pubertal, proportion[2]	3.92 (0.981)	2.94 (0.950)	1.48 (0.815)	<0.01	–1.22 ± 0.46	0.70	0.24 ± 0.61
13 mo pubertal, proportion[2]	4.34 (0.987)	4.23 (0.986)	2.09 (0.890)	0.02	–1.13 ± 0.48	0.16	1.02 ± 0.72

[1]Puberty results based on logit transformation.

[2]Transformed pubertal rate = eLSMean Estimate/(1 + eLSMean^{Estimate}), in which LSMean^{Estimate} is the Least Squares.

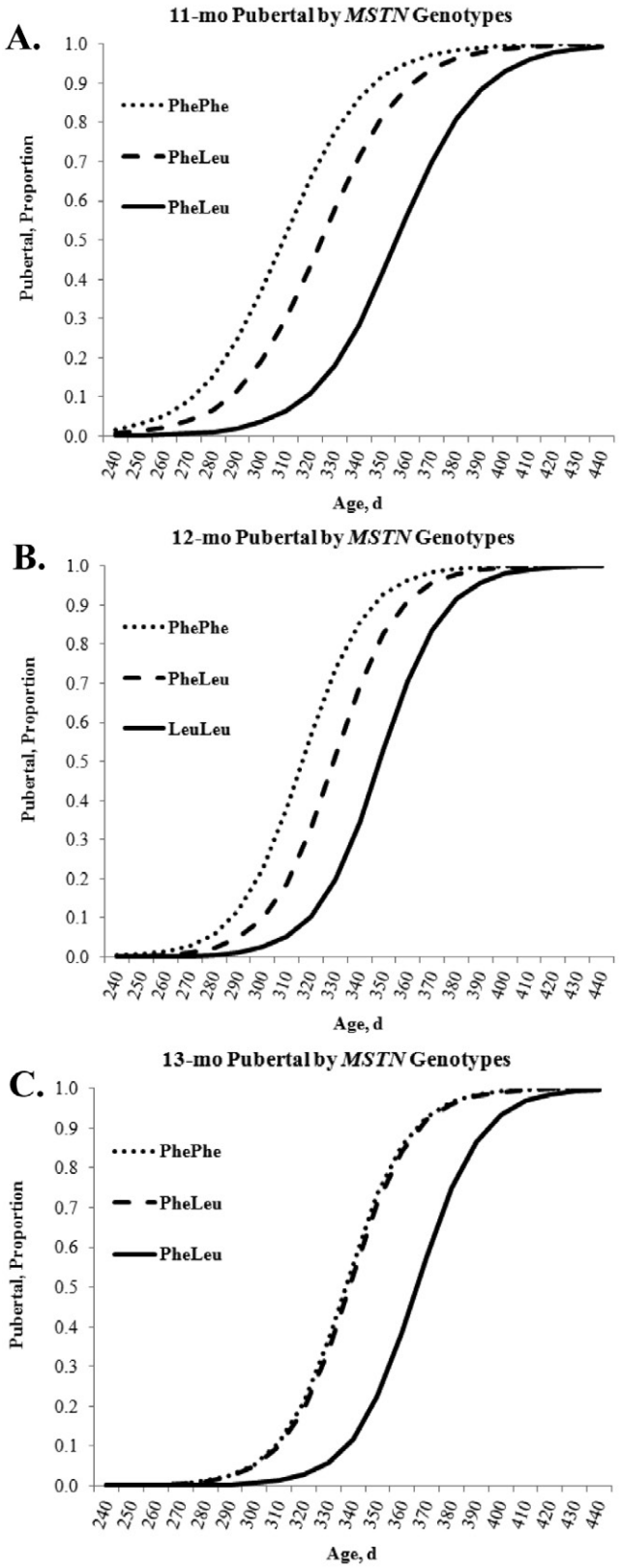

Figure 1. Least squares means for *MSTN* FF, Phe:Leu, and Leu:Leu genotypes on age response curves for ovulation and attainment of puberty (A) at 11 mo ($P < 0.001$), (B) at 12 mo ($P = 0.03$), and (C) at 13 mo ($P = 0.03$).

Table 5. Significance (*P*-value) of fixed effects on first calf performance traits

Trait	Calf birth year	Dam devel-opment	Calf age	Calf sex	Dam *MSTN*	Dam *CAPN1*	Calf *MSTN*
Weaning rate	0.57	0.91	NA[1]	NA	0.77	0.51	NA
Julian calving date	0.79	0.10	NA	0.55	0.15	0.32	NA
Birth BW	0.01	0.18	0.86	<0.001	0.54	0.04	NA
Birth BW	0.02	0.14	0.94	<0.001	0.78	0.04	0.17
Weaning BW	<0.001	0.19	NA	0.01	0.35	0.91	NA
Age-adjusted weaning BW	0.02	0.29	<0.001	<0.01	0.71	0.90	NA

[1]NA indicates this effect was not included in the model for this trait.

the uterus or the ovaries on d 4 of the estrous cycle between the 2 genotypes ($P \geq 0.43$; Table 7). The number of small and medium antral follicles also did not differ between the 2 genotypes ($P \geq 0.22$). Histological analysis of the ovaries revealed no difference in the number of microscopic primordial follicles per section between the 2 genotypes ($P = 0.37$; Table 8). Furthermore, there was no difference in the number of primary, secondary, or tertiary follicles per section ($P \geq 0.14$). The characteristics of the largest follicle on d 4 after estrus did not differ between the 2 genotypes ($P \geq 0.13$; Table 9). For real-time RT-PCR analysis, the raw threshold cycles for *YWHAG* did not differ between genotypes ($P > 0.10$), confirming the reliability of *YWHAG* as an internal reference gene in this model system. Real-time RT-PCR analysis revealed no difference in the abundance of chemerin mRNA in adipose tissue between the 2 *MSTN* genotypes ($P = 0.55$; Fig. 3).

DISCUSSION

The most significant finding of the current study was that the Leu allele of the *MSTN* F94L polymorphism was associated with a delay in the onset of puberty. However, this delay in puberty did not result in an increase in calving day or a decrease in pregnancy rates during the first breeding season. This is consistent with the results of Cundiff et al. (2007), who reported that in Cycle VII of the Germplasm Evaluation project, heifers from the Limousin breed (which have a greater frequency of the Leu allele than other breeds in Cycle VII) reached puberty later than other breeds but that this delay in puberty did not result in differences in pregnancy rates among the 7 breeds of heifers that were evaluated. The cause of this delay in onset of reproductive cycles is not clear from the results of the present study, because the mechanism of action of the *MSTN* F94L polymorphism is unknown.

A disassociation of age at puberty with calving day has been observed in a number of studies (Martin et al.,

Table 6. Least squares means for *CAPN1* haplotype effects on first calf performance traits and significance (*P*-value) of additive and dominance modes of inheritance

	CAPN1 genotypes			Additive effect of GT		Dominance effect of GT	
Trait	CC:CC	CC:GT	GT:GT	*P*-value	Estimate ± SE	*P*-value	Estimate ± SE
Birth BW, kg	35.7	34.7	32.7	0.01	–1.50 ± 0.59	0.55	0.47 ± 0.79
Birth BW,[1] with calf *MSTN* genotype, kg	35.9	35.0	32.9	0.01	–1.50 ± 0.59	0.43	0.63 ± 0.79

[1]Adjusted for calf *MSTN* genotype.

2007; Funston et al., 2012). As stated above, the Leu allele of *MSTN* was associated with a delay in onset of puberty but did not cause a significant delay in the calving day. Conversely, there are cases where conception occurs earlier in the breeding season without an associated decrease in the age at puberty. Increased concentrations of energy or protein provided to the dam during the third trimester caused the daughters born to those dams to conceive earlier in their first breeding season without changing age at puberty (Martin et al., 2007; Cushman et al., 2014). Therefore, while the attainment of puberty is a limiting step for early conception in heifers, it is clear that there are other mechanisms contributing to the timing of conception during the first breeding season, and heifers that reach puberty at an older age can still conceive early in their first breeding season as occurred in the present study.

We observed no influence of the *CAPN1* haplotype on age at puberty in heifers in the current study. This is in agreement with Collis et al. (2012), who reported no influence of *CAPN1* on age at puberty; however, in that study, there was an influence of *CAPN1* on postpartum anestrous interval. Therefore, as these heifers continue in the production herd as mature cows, it will be interesting to determine if *CAPN1* is influencing postpartum reproductive performance or length of productive life. The *CAPN1* genotype of a heifer influenced the birth BW of her first calf, with the haplotype previously associated with decreased tenderness (GT; White et al., 2005) also associated with a reduced first calf birth BW. This is an important relationship to be identified. Beef cattle breeders have been selecting parents for calves with moderate birth BW. At the same time, beef cattle breeders are interested in selecting cattle for more tender eating experiences. Caution will be needed, because use of the *CAPN1* marker to improve tenderness could result in an unwanted increase in birth BW based on the antagonistic relationships observed in the present study. However, further research will be necessary to determine if this association has a biological basis.

The delay in the onset of puberty does not appear to be due to differences in development of the anterior pituitary gland, the source of gonadotropins. There was no difference in anterior pituitary weights of pubertal heifers from the 2 homozygous *MSTN* genotypes. Interestingly, there was an increase in anterior pituitary weight as antral follicle count of the ex vivo ovaries increased, whereas Mossa et al. (2010) reported no difference in anterior pituitary weights between dairy cows with high or low numbers of antral follicles. The weights of the anterior pituitary glands reported in the current study are much less than those reported by Mossa et al. (2010), indicating that growth

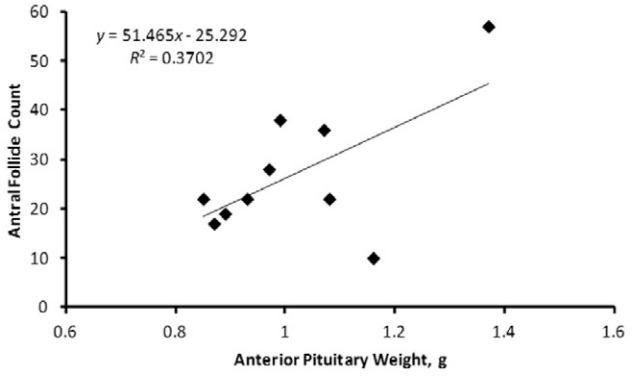

Figure 2. Relationship between anterior pituitary weight and surface follicle counts in yearling beef heifers. There was a positive linear relationship such that as the anterior pituitary weight increased the number of surface follicles tended to increase ($P = 0.06$).

Table 7. Reproductive tract traits for heifers homozygous for the Phe allele or the Leu allele of the F94L polymorphism of *MSTN*

	MSTN genotype		
Trait	Phe:Phe	Leu:Leu	*P*-value
Heifers	5	5	–
Reproductive tract weight, g	196.0 ± 15.9	177.5 ± 17.7	0.46
Ovarian length,[1] mm	26.9 ± 1.8	28.0 ± 1.8	0.68
Ovarian height,[2] mm	16.5 ± 1.0	16.0 ± 1.0	0.76
Uterine horn diameter,[3] mm	22.6 ± 2.6	24.1 ± 2.6	0.69
Endometrial diameter,[4] mm	17.2 ± 2.0	19.6 ± 2.0	0.43
Small follicles[5]	29.4 ± 6.2	20.6 ± 6.2	0.34
Medium follicles[6]	1.8 ± 0.3	2.4 ± 0.3	0.22
Luteal weight, g	1.2 ± 0.4	1.1 ± 0.4	0.96

[1]Average length of the 2 ovaries within a heifer.

[2]Average height of the 2 ovaries within a heifer.

[3]Average diameter of the 2 uterine horns, 1 cm anterior to the uterine bifurcation.

[4]Average diameter of the endometrium, 1 cm anterior to the uterine bifurcation.

[5]Number of surface follicles <5 mm in diameter.

[6]Number of surface follicles 5 to 10 mm in diameter.

Table 8. Numbers of preantral follicles per section in 5 histological sections taken from a cross-section in the middle of the ovary of yearling heifers

	MSTN genotype		
Follicle class	Phe:Phe	Leu:Leu	*P*-value
Heifers	5	5	–
Primordial follicles[1]	88.3 ± 18.3	65.4 ± 18.3	0.37
Primary follicles[2]	28.9 ± 4.1	20.2 ± 4.1	0.14
Secondary follicles[3]	9.0 ± 1.1	8.8 ± 1.1	0.92
Tertiary follicles[4]	5.8 ± 2.1	6.8 ± 2.1	0.75

[1]Oocyte surrounded by a single layer of squamous pregranulosa cells.

[2]Oocyte surrounded by a single layer of cuboidal granulosa cells.

[3]Oocyte surrounded by 2 or more layers of granulosa cells.

[4]Oocyte surrounded by multiple layers of granulosa cells with an antrum.

Table 9. Comparison of largest medium follicle on d 4 after estrus between heifers homozygous for F94L genotypes in the *MSTN* gene

	MSTN genotype		
Follicle trait	Phe:Phe	Leu:Leu	*P*-value
Heifers	5	4	–
Follicular fluid volume, µL	316.7 ± 51.3	181.3 ± 62.8	0.13
Follicular fluid estradiol, ng/mL	168.1 ± 48.9	114.9 ± 59.9	0.51
Granulosa cell weight, g	0.07 ± 0.05	0.02 ± 0.06	0.56
Theca cell weight, g	0.11 ± 0.03	0.10 ± 0.03	0.88

of the anterior pituitary gland continues into maturity. Therefore, the positive relationship between antral follicle count and anterior pituitary weight in these growing heifers might indicate a mechanistic role for the anterior pituitary gland in antral follicle counts in growing heifers. Alternatively, the greater anterior pituitary gland weights could be a product of increased numbers of follicles, but this seems unlikely.

Chemerin gene expression was of interest from 2 perspectives. First, chemerin suppresses steroidogenesis and proliferation of granulosa cells and inhibits steroidogenesis in theca cells (Reverchon et al., 2012), and thus, there is a direct potential link between chemerin expression and puberty. An increased level of expression would be consistent with an adipose-mediated suppression of steroidogenesis contributing to delay of puberty. Second, chemerin mRNA abundance is negatively correlated with BCS in peripubertal heifers (Lindholm-Perry et al., 2012), and body condition can influence age at puberty. Despite a reduction of fat depth in steers carrying the Leu allele, *MSTN* genotype did not affect chemerin mRNA abundance in adipose tissue. This indicates that adipose-mediated effects on puberty are not the primary mechanism of action of this allele. Moreover, the consistent concentrations of estradiol in follicular fluid of the largest follicle (d 4 of the estrous cycle) across genotypes suggest that allelic effects do not act through impacts on steroidogenesis.

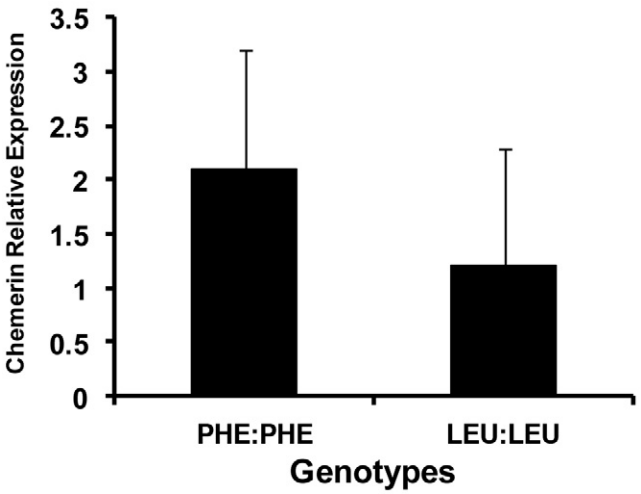

Figure 3. Relative amounts of chemerin mRNA in adipose tissues homozygous for the Phe allele or homozygous for the Leu allele of the *MSTN* F94L polymorphism. There was no difference in relative amounts of chemerin mRNA between the 2 genotypes (*P = 0.55).*

Skinner et al. (2008) reported the presence of *MSTN* mRNA in bovine granulosa cells in a microarray study that examined changes in transcript profiles in the granulosa and theca cells during the process of follicular development. Messenger RNA for *MSTN* was at its greatest amount in the granulosa cells in small (<5 mm) antral follicles and decreased in medium (5–10 mm) and large (>10 mm) antral follicles, indicating that, like other TGF-β superfamily members, *MSTN* may regulate granulosa cell function during the process of follicular development and selection. Furthermore, in women with polycystic ovary syndrome, serum myostatin concentrations were positively related to serum androgen concentrations and negatively related to serum follistatin concentrations (Chen et al., 2012), indicating an important role for *MSTN* in normal ovarian function in mammalian females. However, there was no difference in weight of the granulosa cell pellet, follicular fluid volume, or theca weight between the 2 genotypes, indicating that the change from a Phe to a Leu at the 94th amino acid of *MSTN* is not causing a change in function that alters follicular development.

There is mounting evidence indicating an antagonistic relationship between production traits and age at puberty in beef heifers (Johnston et al., 2009; Collis et al., 2012; present study). Therefore, while the delay in puberty in the current study did not impact heifer reproductive performance, care should be taken when using genetic markers to improve production traits. Even if individual alleles favorable for production traits do not detectably interfere with reproduction, it is possible that combinations of alleles selected to increase carcass productivity could interact in a way

detrimental to reproduction. In support of this, many of the genes involved in growth and development are also involved in development of the reproductive tract. It will be important to understand the mechanisms of action and the interactions of these genes to ensure that fertility in the cow herd is not negatively impacted by using these markers to improve production traits.

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